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[Title of invention] **Fibroblast Growth Factor FGF-10**
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[Document name] Detailed Description
[Title of invention] Fibroblast Growth Factor FGF-10

[Patent claims]

[Claim 1]

Recombinant DNA containing a nucleotide sequence encoding the polypeptide fibroblast growth factor whose amino acid sequence is represented by sequence number 1 or 2, or a nucleotide sequence complementary to the above nucleotide sequence.

[Claim 2]

The DNA described under Claim 1, containing the nucleotide sequence represented by sequence number 3 or 4 or a nucleotide sequence complementary to the above nucleotide sequence.

[Claim 3]

Expression vector containing the DNA described under Claim 1.

[Claim 4]

Transformant obtained by introducing the expression vector described under Claim 3 into a host.

[Claim 5]

The transformant described under Claim 4, in which the host is an animal cell or E. coli.

[Claim 6]

Method for producing recombinant fibroblast growth factor, characterized by use of the transformant described under Claim 4.

[Claim 7]

Recombinant fibroblast growth factor that is a polypeptide containing the amino acid sequence represented by sequence number 1 or 2 or their major portions.

[Claim 8]

Recombinant fibroblast growth factor that is a polypeptide containing the amino acid sequence represented by sequence number 1 or 2 or their major portions, characterized by the fact that it is produced by the transformant described under Claim 5 and that has cell-growth-promoting activity.

[0001]

[Field in the Industry]

The present invention relates to a novel fibroblast growth factor (in the following, abbreviated as FGF) and to a method for producing the same by recombination.

[0002]

[Prior Art]

FGF was discovered in the 1970's as an angiogenic factor. Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) have been studied and their structures and wide-ranging cell-growth-promoting actions have been elucidated [D. Gospodarowics et al.: Nature Vol. 249, page 123 (1974); Burgess, W. H. and Maciag, T.: Annu. Rev. Biochem. Vol 58, pages 575-606 (1989); Suzuki, F.: Clinical Calcium, Vol. 4, pages 1516-1517 (1994)]. Currently, there are a total of 9 FGF species. They all have been cloned and their structures are known [Cell, Vol. 27, No. 9, pages 341-344 (1995)]. Existence of additional FGF species has been suggested.

[0003]

On the other hand, based on their wide-ranging cell-growth-promoting actions, aFGF and bFGF have been evaluated for their possible applications as promising therapeutic agents for the treatment of metabolic diseases of the nervous, cardiovascular and bone systems. However, the usefulness in clinical application so far has not been established. The same evaluation of novel FGF species is desired.

[Problems to be Solved by the Invention]

The objective of the present invention is to provide a method for industrial production of a recombinant protein of a novel FGF after identifying then analyzing the gene.

[0004]

[Means of Solving the Problems]

The inventors actively investigated DNAs of unknown FGF species. As a result, they successfully obtained a DNA of a totally novel FGF (in the following, abbreviated as FGF-10), thereby achieving the present invention.

As described below, the present invention relates to the DNA encoding FGF-10, an expression vector containing the DNA, a transformant, a method for producing a recombinant protein by using the transformant, and the recombinant protein.

[0005]

(1) A nucleotide sequence encoding a fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or 2, or a recombinant DNA containing a nucleotide sequence complementary to the above sequence.

(2) The DNA described under Claim 1, containing the nucleotide sequence represented by sequence number 3 or 4 or a nucleotide sequence complementary to the above sequence.

(3) An expression vector containing the DNA described under (1).

(4) A transformant obtained by introducing the expression vector described under (3) into a host cell.

(5) The transformant described under (4), in which the host cell is an animal or E. coli cell.

(6) A method for producing a recombinant fibroblast growth factor, characterized by use of the transformant described under (4).

(7) A recombinant fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or 2 or their main portions.

(8) The recombinant fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or 2 or its main portions, characterized by the fact that it is produced by the transformant described under (5) and has cell-growth-promoting activity.

[0006]

In the present Detailed Description, definition of the technical terms is as follows.

FGF-10: A mammalian fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or its major portions. The major portions include the amino acid sequence of mature protein after the signal (pre)sequence or prosequence is deleted from the above sequence. Specifically, they are the amino acid sequences of 179 amino acid residues from glutamine (Gln)-37 to serine (Ser)-215 in sequence number 1 and 172 amino acid residues from glutamine (Gln)-38 to serine (Ser)-208 in sequence number 2.

By techniques well known in this field, proteins can be produced by deletion, substitution or addition of some amino acid residues on the amino acid sequence represented by sequence number 1 or 2 or their major portions. Based on random screening or knowledge on sequence modification studies on other proteins of the FGF family, modified proteins with identical physiological properties to that of the present FGF-10 can be produced. As long as these modified proteins have the fibroblast growth factor activity, they are covered by the present patent. Moreover, FGF-10 has two glycosylation sites: Asn-Ser-Ser of residue numbers 50-52 and Asn-Thr-Ser of residue numbers 203-205 in the amino acid sequence represented by sequence number 1 or Asn-Ser-Ser of residue numbers 51-53 and Asn-Thr-Ser of residue numbers 196-198 in the amino acid sequence represented by sequence number 2. In general, the biological activity is not dependent on glycosylation. By selection among known hosts, sugar chains can be changed or removed. The proteins with modified glycosylation are also covered by the present patent as long as they have the fibroblast growth factor activity. Activity of fibroblast growth factor: At least one of the various biological activities of FGF family including cell-growth-promoting activity such as cell-growth-stimulating activity, hemopoietic-progenitor-growth-stimulating activity, angiogenic activity, etc., differentiation-modulating activity such as cell differentiation-inducing activity, extracellular-matrix-modifying activity, etc., nerve-cell survival-maintaining activity, etc. [Clinical Biochemistry, Vol. 38, No. 11, pages 219-221 (1994 Supplement Issue)].

[0007]

In the following, the present invention is described in more detail.

[Preparation of FGF-10 gene]

The DNA encoding FGF-10 of the present invention can be obtained by known genetic engineering techniques. Specifically, mRNA can be isolated from animal tissues or cells, then double-stranded cDNA can be synthesized. The cDNA can be amplified by PCR using primers and the sequence can be determined. For all these experiments, special kits are commercially available. Although there is no special limitation to tissues and culture cells as a source of mRNA, rat embryo at about day 14 is used preferably. Since the expression level of the mRNA is relatively higher in the lung and articular tissues, lung cells and culture cells derived from bone/cartilage also can be used. Use of commercially available poly(A) + RNA from adult human lung (from Clontech) is simple and therefore preferable.

[0008]

It can also be cloned from cDNA or genomic DNA libraries from various species by using appropriate sequences from the DNA sequence encoding FGF-10 disclosed in the present Detailed Description as DNA probes.

DNA library is prepared as follows by standard procedures. 1. Lyophilized animal tissue is treated with RNase and protease, then high molecular weight DNA is obtained by precipitation. DNA extracts are commercially available (from Clontech, etc.). 2. By partial digestion with restriction enzyme (EcoRI, etc.), DNA fragments are obtained by ethanol precipitation. 3. The DNA fragments are inserted into λ phage by using DNA ligase. 4. By using commercially available in vitro packeting kit, packeting is performed, thereby obtaining a DNA library.

DNA probes are selected based on highly distinct sequences from the DNA sequences encoding the FGF proteins disclosed in the present Detailed Description. They are chemically synthesized, then labeled with ^{32}P, etc.

[0009]

[Preparation of FGF-10 protein]

As the expression vector containing the FGF-10 cDNA thus obtained, plasmid or phage is selected, that can be amplified in appropriate host cells of E. coli, Bacillus subtilis, yeast, animal or insect. For example, the vector can be pBR322 or pBR325 derived from E.

coli [Gene, Vol. 4, page 121 (1978)], pUB110 derived from Bacillus subtilis [Biochem. Biophys. Res. Commun. Vol. 112, page 678 (1983)], pCDM8 that is preferable for COS cells, etc. For the insertion of cDNA into plasmid, standard procedures are described in Molecular Cloning by T. Maniatis et al., Cold Spring Harbor Lab, page 239 (1982).

[0010]

The host cells are transformed by the introduction of the vector. There is no special limitation to the host cells, as long as they can produce FGF-10. Typical examples include bacteria such as E. coli, Bacillus subtilis (Bacillus bacteria), etc., yeast such as Saccharomyces, Torula, pikia(?), etc., animal cells such as CHO cells, NSO cells, etc. Not only cultured insect, fungus and plant cells, but also insects, mammals and plants containing the gene for the target protein are included in the hosts.

[0011]

Desired clones are selected from the transformants by known methods such as colony hybridization method [Gene, Vol. 10, page 63 (1980)] and DNA sequence determination method [Proc. Natl. Acad. Sci. USA, Vol. 74, page 560 (1977)]. Besides, clones also can be selected by transient expression in COS cells, followed by evaluation of the biological activity in the culture supernatants.

[0012]

The biological activity of the expressed FGF-10 can be easily detected by standard methods. For example, it can be evaluated by assaying the growth-promoting activity for epithelial cells such as the known cell line FRSK.

[0013]

The plasmid containing the cloned DNA can be used directly, or after cut with restriction enzyme then inserted into an expression vector appropriate for selected host. FGF-10 protein can thus be produced in large quantities. There is no special limitation to expression method. All known techniques in this field can be used. For example, fusion expression, secretion expression or direct expression using bacteria, or expression using eukaryotic cells can be selected appropriately.

[0014]

The FGF-10 protein thus produced by recombinant technology can be purified by purification techniques generally used in the biochemical field. For example, appropriate combinations of ion-exchange chromatography, gel filtration, reverse phase HPLC, ammonium sulfate precipitation, ultrafiltration, SDS-PAGE, etc. can be used. For FGFs, particularly affinity chromatography using heparin, etc. as a ligand, antibody column chromatography, etc. are preferably used for large scale purification. Antibodies against FGF-10 protein, both monoclonal and polyclonal, can be produced by known techniques. Specific antibodies against FGF-10 can be used not only for antibody column, but also for immunochemical quantitative assays such as ELISA, etc.

[0015]

[Mechanisms]

The FGF-10 protein produced by the above methods has various biological activities including cell-growth-promoting activity and thus can be used as a wound-healing-promoting agent, circulation-deficiency-treating agent, nerve-cell-survival-maintaining agent, hair-growth-promoting agent, etc. for medical applications. In particular, since its expression in chondral tissues of adult mammals, its applications as a bone disease-treating agent for the treatment of fractures, etc. and as a therapeutic agent for the treatment of injuries of chondral and connective tissues are possible. Moreover, it also can be used as a reagent for research on cell-growth promotion.

[0016]

[Practical Examples]

In the following, the present invention is further described in detail by the way of practical examples. Nevertheless, the present invention is not to be limited to the examples.

[Practical Example 1]

Structural analysis of FGF-10 gene

Preparation of rat DNA library

From a whole, 14 day old Wistar rat embryo, mRNA was prepared by standard procedures [Chomoczynski et al.: Anal. Biochem. Vol. 162, pages 156-159 (1987)]. The rat embryo mRNA was used as template to prepare rat embryo cDNA using random primer (6mer) as primer and Moloney murine leukemia virus reverse transcriptase. Specifically, rat

embryo poly(A)+ RNA (5 μg) was incubated in a solution containing 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals) and 0.5 μg of random primer (6mer) at 37°C for 60 minutes, thereby obtaining the cDNA.

[0017]

Preparation of primers common between FGF-3 and FGF-7

By comparing the amino acid sequences of 7 known human FGF species, 2 regions of amino acid sequences common between FGF-3 and FGF-7 (Tyr-Leu-Ala-Met-Asn-Lys and Tyr-Asn-Thr-Tyr-Ala-Ser) were selected, and 2 FGF primers as shown in Figure 1 were prepared.

[0018]

Amplification of rat FGF family DNAs

The rat embryo cDNA was used as template. The above 2 FGF primers and Taq DNA polymerase were used to amplify FGF family DNAs by the polymerase chain reaction (PCR) method. Specifically, a reaction solution (25 μL) containing an appropriate amount of cDNA, 0.05 unit/μL of Taq DNA polymerase (Wako Pure Chemicals) and 5 pmol/μL of the above sense or antisense primer was subjected to 30 cycles of PCR. After the reaction, the solution was applied to 8% polyacrylamide gel electrophoresis, and the fraction with the desired size (about 110 bp) was eluted electrophoretically.

[0019]

Screening of rat FGF family DNAs

The FGF family DNAs thus amplified by using FGF primers were inserted into pGEM-T DNA vector (Promega). The resultant recombinant vector was transformed into E. coli (strain XL1-blue), thereby obtaining DNA clones. DNA Sequencer 373A (Applied Biosystems, Inc.) was used for the analysis of cDNA sequence.

By determining the nucleotide sequences of all the DNA clones, besides cDNAs for FGF-3 and FGF-7 that were known, a novel FGF cDNA encoding a peptide with similar amino acid sequence to known FGF a family of peptides (about 50% similarity) was isolated. The clone was named FGF-10.

[0020]

Structural analysis of entire coding region of rat FGF-10 cDNA

Based on the partial structure of FGF-10 cDNA identified in the above experiments, several primers were prepared. The entire coding region was obtained by using Rapid Amplification of cDNA Ends (RACE) method [Frohman, PCR Protocols - A Guide to Methods and Applications, Academic Press, pp. 28-38 (1990)]. Details are described under (1)-(6).

[0021]

(1) Based on the partial structure of FGF-10 cDNA, primers A-D (Figure 2, sequence numbers 5, 6, 7 and 8) were prepared. In addition, primers X and Y (Figure 2, sequence numbers 9 and 10) were prepared by the RACE method. (2) Random hexaoligonucleotide was used as primer to synthesize cDNA with reverse transcriptase using rat embryo mRNA as template. A poly(A) sequence was added to the 3'-terminus with 3'-deoxynucleotidyl transferase in the presence of deoxyadenine triphosphate. The cDNA thus prepared was used as template to perform PCR using primers B and X. Furthermore, primers A and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named pFGF-10 (5'). (3) cDNA was synthesized with reverse transcriptase using the rat embryo mRNA as template and primer X. The cDNA thus produced was used as template to perform PCR using primers C and Y. Furthermore, primers D and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named pFGF-10 (3'). (4) Based on the most upstream nucleotide sequence of pFGF-10 (5') and the most downstream nucleotide sequence of pFGF-10 (3'), primers E and F, respectively, were prepared (Figure 2, sequence numbers 11 and 12). (5) Rat embryo mRNA was used as template to synthesize single-stranded cDNA with reverse transcriptase using oligo(dT) as primer, that was then used as template to perform PCR using primers E and F. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined, thereby obtaining clones with a nucleotide sequence containing the most upstream nucleotide sequence of pFGF-10 (5') and

the most downstream nucleotide sequence of pFGF-10 (3'). Among them, 1 clone was selected, named pFGF-10. FGF-10 cDNA containing entire coding region, carried by the plasmid, was analyzed. (6) Taken together, the nucleotide sequence of sequence number 3 (804 bp) was determined.

[0022]

Determination of entire amino acid sequence of rat FGF-10

Based on the nucleotide sequence of FGF-10 cDNA obtained in the above experiments, it was known that the open reading frame of FGF-10 cDNA consists of 645 bp and that FGF-10 is a novel FGF consisting of 215 amino acids represented by sequence number 1. Amino acid sequence analysis revealed that it is a secreted protein with a signal peptide at the N-terminus. The mature protein is the polypeptide of 179 amino acid residues between positions 37-215. The Asn-Ser-Ser of residue numbers 50-52 and Asn-Thr-Ser of residue numbers 203-205 are glycosylation sites, and therefore FGF-10 can be glycosylated.

[0023]

[Practical Example 2]

Expression of rat FGF-10 in mammalian cells

Construction of plasmids

The plasmid pFGF-10 (Figure 3) was digested with SphI and PstI, then a fragment containing the full length cDNA was separated by polyacrylamide gel electrophoresis. The fragment was ligated into pUC19 that had been digested with SphI and PstI, then transformed into E. coli strain JM109, thereby obtaining the plasmid pUC-F10 containing FGF-10 cDNA. pUC-F10 was digested with HindIII and XbaI to cut off a fragment containing FGF-10 cDNA, which was then ligated into the mammalian cell expression vector pCDM8 that had been digested with HindIII and XbaI, followed by transformation into E. coli strain MC1061/P3, thereby obtaining the plasmid pCDM8-F10SP containing FGF-10 cDNA downstream of CMV promoter.

On the other hand, since the nucleotide sequence upstream of the deduced translation start site in the FGF-10 cDNA was different from the Kozak consensus sequence, the translation efficiency of the mRNA was not considered to be high. Accordingly, to increase the translation efficiency, it was decided to perform mutation to convert the sequence

upstream of the deduced translation start site to the Kozak consensus sequence [M. Kozak, The Journal of Cell Biology, Vol. 108, pages 229-241 (February, 1989)].

PCR was used to perform the mutation. pFGF-10 was used as template and a sense primer having, as shown in Figure 4, the HindIII site at the 5'-end and a Kozak consensus sequence and an antisense having an XbaI site at the 5'-end was used (for reaction conditions, see Figure 4).

After the reaction, the PCR product was subjected to phenol-chloroform treatment, ether treatment and then ethanol precipitation. After digestion with HindIII and XbaI, a fragment with about 700 bp was isolated by polyacrylamide gel electrophoresis. The fragment was ligated into the mammalian cell expression vector pCDM8 that had been digested with HindIII and XbaI. The vector was transformed into E. coli strain MC1061/P3. Among the resultant colonies, 4 clones were selected and the nucleotide sequences were analyzed using a DNA Sequencer (Perkin Elmer model 373).

The results showed that, in all the clones, the sequence upstream of the deduced translation start site was converted to the Kozak consensus sequence, and that there was no mutation on the amino acid sequence encoded. One clone was selected among these clones, named pCDM8-F10HX.

[0024]

[Practical Example 3]

Transformation of rat FGF-10-expressing plasmids in COS-1 cells

The rat FGF-10-expressing plasmids constructed in Practical Example 2, pCDM8-F10SP and pCDM8-F10HX, were prepared in large quantities by standard procedures, and purified by performing cesium chloride density gradient centrifugation twice. The two plasmids and pCDM8 as control were transformed into COS-1 cells by electroporation. The transformed cells were cultured for 24 hours in DMEM containing 10% bovine fetal serum that had been treated by lysine-Sepharose chromatography; then the medium was changed to serum-free DMEM. The culture was further conducted for 96 hours. The culture supernatant thus obtained was centrifuged. The supernatant was stored at -80°C in aliquots.

[0025]

[Practical Example 4]

Confirmation of expression of FGF-10 mRNA in cartilage by in situ hybridization

Preparation of probe: FGF-10 cDNA was inserted into the vector pGEM-T. The plasmid was transformed into E. coli strain JM-109. The bacteria were cultured in a large quantity. FGF-10 cDNA was highly purified by using Flexi Prep Kit from Pharmacia. The DNA sequence was confirmed by using a Perkin Elmer 373A/DNA Sequencer. The cRNA probe was prepared by using the DIG/RNA Labeling Kit (SP6/T7) from Boehringer.

[0026]

Preparation of tissue slice: A 3 weeks old female Wistar rat was sacrificed. A thigh bone and tibia with the joint in its original shape was collected. After the soft tissues had been removed, the specimen was trimmed into appropriate sizes then immediately soaked in fixing solution (4% paraformaldehyde). After fixing at 4°C overnight, dehydration was performed, followed by soaking in ash-removing solution (10% EDTA and 15% glycerol in PBS) for 4-5 days (the solution was replaced with fresh solution every day). The knee joint was trimmed to a thickness of about 2 cm, followed by soaking in OCT compound and frozen with liquid nitrogen. A cryostat was used to obtain 10 μm thick joint tissue slices, which were then mounted on silane-coated glass slide.

Hybridization: After the above joint tissue slides were subjected to pretreatment (digestion with proteinase K, inactivation of endogenous alkaline phosphatase with 0.2 M HCl, and acetylation with 0.1 M TEA and 0.25% acetic anhydride), they were dehydrated with ethanol. The above probe was diluted tenfold with hybridization solution (50% formamide, 10 mM Tris-HCl/pH 7.6, 200 μg/mL tRNA, 1 x Denhardt's solution, 10% Dextran sulfate, 600 mM NaCl, and 0.25% SDS), then 50 μL per slide was used. The sample was covered with a small sheet of parafilm. The hybridization was performed at 50°C for 16 hours. The excess probe was digested with RNase A. After washing with SSC, antibody binding and color development were performed.

[0027]

Antibody binding and color development: After the probe was washed off, the slide was soaked in blocking solution for 60 minutes. Alkaline-phosphatase-labeled anti-digoxigenin antibody (anti-digoxigenin-AP:Fab fragment, from Boehringer-Mannheim) was

applied onto the slide. After incubation at 37°C for 1 hour, the antibody solution was washed off. NBT, X [sic] phosphate was added, followed by incubation at 37°C for color development (12 hours). After the color was developed, the slide was soaked in color development stopping solution (10 mM Tris-HCl/pH 7.6, 1 mM EDTA/pH o.0). After washing with distilled water, the slide was sealed with water.

Results: As shown in Figure 5 (A) and (B), color development was recognized in chondral cells. Since FGF-10 mRNA is expressed in chondral cells, it is suggested that FGF-10 may be a factor involved in wound healing of bone and cartilage.

[0028]

[Practical Example 5]

Evaluation of cell-growth-promoting activity in FRSK cells

Cell culture: The rat epithelial cell line FRSK was cultured in culture flask with a culture area of 75 cm^2 in 15 mL of F-12 medium containing 10% of bovine fetal serum at 37°C in an atmosphere of 5% carbon dioxide/95% air. The cells were split at 1/10 once a week.

Expression of FGF-10 protein: FGF-10 was transiently expressed in COS-1 cells (see Practical Example 3). The culture supernatant was used in the following assays (in the following, the culture supernatants obtained from pCDM8-F10SP, pCDM8-F10HX and the control pCDM8 are represented by FGF-10/Sp, FGF-10/Hx and Bq, respectively).

DNA synthesis assay (tritium-labeled thymidine incorporation): The cells were cultured until subconfluence, then collected by trypsin treatment. A cell suspension at 10,000 cells/mL in the above medium was prepared, then distributed at 100 μL/well in a 96-well plate. The plate was incubated at 37°C in an atmosphere of 5% carbon dioxide/95% air. The medium was replaced with 100 μL of fresh medium once every 2 days. After 7 days of culture, the medium was replaced with 100 μL of F-12 medium containing 0.1% of bovine fetal serum. After 24 hours, 25 μL of the COS supernatant was added. After 18 hours, culture at 37°C in an atmosphere of 5% carbon dioxide/95% air, 20 μL of F-12 medium containing 0.2 μCi of tritium-labeled thymidine was added, followed by incubation under the same conditions. After 4 hours, the medium was removed, and 50 μL of 2 N

NaOH was added, followed by standing for 30 minutes to kill the cells. After neutralization with 1 N HCl, the cells were recovered with a cell harvester, and counted in Betaplate.
Results: As shown in Figure 6, compared to the control (Bq, 100%), the FGF-10 expression samples (Sp and Hx), both greatly enhanced the incorporation of tritium-labeled thymidine into FRSK cells (286% and 501%, respectively). Thus, it is shown that FGF-10 is a factor promoting the growth of epithelial cells.

[0029]

[Practical Example 6]

Structural analysis of human FGF-10 gene

Preparation of human DNA library

Commercially available human lung poly(A)+ RNA (from Clontech, Cat. No. 6524, from the whole lung of a male adult) was used as template to prepare human lung cDNA using random primer (6mer) as primer and Moloney murine leukemia virus reverse transcriptase. Specifically, human lung poly(A)+ RNA (5 μg) was incubated in a solution containing 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals) and 0.5 μg of random primer (6mer) at 37°C for 60 minutes, thereby obtaining the cDNA.

[0030]

Preparation of primers for amplifying human FGF-10 DNA and amplification of human FGF family DNAs

The two FGF primers used in Practical Example 1, shown in Figure 1 (Tyr-Leu-Ala-Met-Asn-Lys and Tyr-Asn-Thr-Tyr-Ala-Ser), were selected and used for the amplification of human FGF-10 DNA.

Human lung cDNA was used as template. The above 2 FGF primers and Taq DNA polymerase were used to amplify FGF family DNAs by the polymerase chain reaction (PCR) method. Specifically, a reaction solution (25 μL) containing an appropriate amount of cDNA, 0.05 unit/μL of Taq DNA polymerase (Wako Pure Chemicals) and 5 pmol/μL of the above sense or antisense primer was subjected to 30 cycles of PCR. After the reaction, the solution was applied to 8% polyacrylamide gel electrophoresis, and the fraction with the desired size (about 110 bp) was eluted electrophoretically.

[0031]

Screening of human FGF family DNAs

The FGF family DNAs thus amplified by using FGF primers were inserted into pGEM-T DNA vector (Promega). The resultant recombinant vector was transformed into E. coli (strain XL1-blue), thereby obtaining DNA clones. DNA Sequencer 373A (Applied Biosystems, Inc.) was used for the analysis of cDNA sequence.

By determining the nucleotide sequences of all the DNA clones, it was found that a cDNA encoding a peptide with identical amino acid sequence to the rat FGF-10 was amplified. The clone was considered to encode human FGF-10.

[0032]

Structural analysis of entire coding region of human FGF-10 cDNA

By the same method as used in Practical Example 1, the entire coding region of human FGF-10 cDNA was cloned and analyzed, thereby obtaining the nucleotide sequence represented by sequence number 4. Details are described under (1)-(6).

[0033]

(1) Based on the partial structure of human FGF-10 cDNA, primers A' and D' (Figure 2, sequence numbers 13 and 14) were prepared. Primers B, C, X and Y (Figure 2, sequence numbers 5, 6, 7 and 8) used in Practical Example 1 were used. (2) Random hexaoligonucleotide was used as primer to synthesize cDNA with reverse transcriptase using the human lung mRNA as template. A poly(A) sequence was added to the 3'-terminus with 3'-deoxynucleotidyl transferase in the presence of deoxyadenine triphosphate. The cDNA thus prepared was used as template to perform PCR using primers A' and X. Furthermore, primers B and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue), thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named phFGF-10 (5'). (3) cDNA was synthesized with reverse transcriptase using the human lung mRNA as template and primer X. The cDNA thus produced was used as template to perform PCR using primers C and X. Furthermore, primers D' and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to

obtain a clone containing the above partial sequences. It was named phFGF-10 (3'). (4) Because the upstream nucleotide sequence of phFGF-10 (5') was identical to the rat gene, primer E (Figure 2, sequence number 12) was used as the 5' side primer. On the other hand, based on the most downstream nucleotide sequence of phFGF-10 (3'), a new primer, F', was prepared as the 3' side primer (Figure 2, sequence number 15). (5) Human lung mRNA was used as template to synthesize single-stranded cDNA with reverse transcriptase using oligo(dT) as primer, which was then used as template to perform PCR using primers E and F'. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined, thereby obtaining clones with a nucleotide sequence containing the upstream nucleotide sequence of phFGF-10 (5') and the farthest downstream nucleotide sequence of phFGF-10 (3'). Among them, 1 clone was selected, named phFGF-10. Human FGF-10 cDNA containing the entire coding region, carried by the plasmid, was analyzed. (6) Taken together, the nucleotide sequence of sequence number 4 (690 bp) was determined.

[0034]

Determination of entire amino acid sequence of human FGF-10

Based on the nucleotide sequence of the cDNA obtained in the above experiments, it was known that the open reading frame of human FGF-10 cDNA consists of 624 bp and that human FGF-10 is a polypeptide consisting of 208 amino acids represented by sequence number 2. Amino acid sequence analysis revealed that it is a secreted protein with a signal peptide at the N-terminus. The mature protein is the polypeptide of 172 amino acid residues between positions 38-208. The Asn-Ser-Ser of residue numbers 51-53 and Asn-Thr-Ser of residue numbers 196-198 are glycosylation sites, and therefore FGF-10 can be glycosylated.

[0035]
[Sequence Table]
sequence number: 1
sequence length: 215
sequence form: amino acid
topology: linear chain
sequence type: peptide
origin
species name: rat
sequence:

Met Trp Lys Trp Ile Leu Thr His Cys Ala Ser Ala Phe Pro His Leu
1 5 10 15
Pro Gly Cys Cys Cys Cys Phe Leu Leu Leu Phe Leu Val Ser Ser Val
20 25 30
Pro Val Thr Cys Gln Ala Leu Gly Gln Asp Met Val Ser Pro Glu Ala
35 40 45
Thr Asn Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Phe
50 55 60
Ser Ser Pro Ser Ser Ala Gly Arg His Val Arg Ser Tyr Asn His Leu
65 70 75 80
Gln Gly Asp Val Arg Trp Arg Lys Leu Phe Ser Phe Thr Lys Tyr Phe
85 90 95
Leu Lys Ile Glu Lys Asn Gly Lys Val Ser Gly Thr Lys Lys Glu Asn
100 105 110
Cys Pro Tyr Ser Ile Leu Glu Ile Thr Ser Val Glu Ile Gly Val Val
115 120 125
Ala Val Lys Ala Ile Asn Ser Asn Tyr Tyr Leu Ala Met Asn Lys Lys
130 135 140
Gly Lys Leu Tyr Gly Ser Lys Glu Phe Asn Asn Asp Cys Lys Leu Lys
145 150 155 160
Glu Arg Ile Glu Glu Asn Gly Tyr Asn Thr Tyr Ala Ser Phe Asn Trp
165 170 175
Gln His Asn Gly Arg Gln Met Tyr Val Ala Leu Asn Gly Lys Gly Ala
180 185 190
Pro Arg Arg Gly Gln Lys Thr Arg Arg Lys Asn Thr Ser Ala His Phe
195 200 205
Leu Pro Met Val Val His Ser
210 215

[0036]
sequence number: 2
sequence length: 208
sequence form: amino acid
topology: linear chain
sequence type: peptide
origin
species name: human
sequence:

Met Trp Lys Trp Ile Leu Thr His Cys Ala Ser Ala Phe Pro His Leu
1 5 10 15
Pro Gly Cys Cys Cys Cys Cys Phe Leu Leu Leu Phe Leu Val Ser Ser
20 25 30
Val Pro Val Thr Cys Gln Ala Leu Gly Gln Asp Met Val Ser Pro Glu
35 40 45
Ala Thr Asn Ser Ser Ser Ser Ser Phe Ser Ser Pro Ser Ser Ala Gly
50 55 60
Arg His Val Arg Ser Tyr Asn His Leu Gln Gly Asp Val Arg Trp Arg
65 70 75 80
Lys Leu Phe Ser Phe Thr Lys Tyr Phe Leu Lys Ile Glu Lys Asn Gly
85 90 95
Lys Val Ser Gly Thr Lys Lys Glu Asn Cys Pro Tyr Ser Ile Leu Glu
100 105 110
Ile Thr Ser Val Glu Ile Gly Val Val Ala Val Lys Ala Ile Asn Ser
115 120 125
Asn Tyr Tyr Leu Ala Met Asn Lys Lys Gly Lys Leu Tyr Gly Ser Lys
130 135 140
Glu Phe Asn Asn Asp Cys Lys Leu Lys Glu Arg Ile Glu Glu Asn Gly
145 150 155 160
Tyr Asn Thr Tyr Ala Ser Phe Asn Trp Gln His Asn Gly Arg Gln Met
165 170 175
Tyr Val Ala Leu Asn Gly Lys Gly Ala Pro Arg Arg Gly Gln Lys Thr
180 185 190
Arg Arg Lys Asn Thr Ser Ala His Phe Leu Pro Met Val Val His Ser
195 200 205

[0037]
sequence number: 3
sequence length: 804 bp
sequence form: nucleic acid
number of chain: double-stranded chain
topology: linear chain
sequence type: cDNA
origin
species name: rat
existing position: 109-753
method for determining characteristic: E
sequence:

```
TAACCAGTAG CCATCACCTC CAGCTGTCTC TTTGCCTCGC ACCAGGTCTT ACCCTTCCAG   60
TATGTTCCTT CTGATGAGAC AATTTCCAGT GCCGAGAGTT TCAGTACA ATG TGG AAG   117
TGG ATA CTG ACA CAT TGT GCC TCA GCC TTT CCC CAC CTG CCG GGC TGC   165
TGT TGC TGC TTC TTG TTG CTC TTC TTG GTG TCT TCC GTC CCT GTC ACC   213
TGC CAA GCT CTT GGT CAG GAC ATG GTG TCA CCG GAG GCT ACC AAC TCC   261
TCT TCC TCC TCC TCT TCC TCC TCC TCG TCC TCT TCC TTC TCC TCT CCT   309
TCC AGC GCG GGG AGG CAT GTG CGG AGC TAC AAT CAC CTC CAG GGA GAT   357
GTC CGC TGG AGA AAG CTG TTC TCC TTC ACC AAG TAC TTT CTC AAG ATT   405
GAA AAG AAC GGC AAG GTC AGC GGG ACC AAG AAG GAA AAC TGT CCG TAC   453
AGT ATC CTA GAG ATA ACA TCA GTG GAA ATC GGA GTT GTT GCC GTC AAA   501
GCC ATT AAC AGC AAC TAT TAC TTA GCC ATG AAC AAG AAG GGG AAA CTC   549
TAT GGC TCA AAA GAA TTT AAC AAT GAC TGT AAA CTG AAA GAG AGG ATA   597
GAG GAA AAT GGA TAC AAC ACC TAT GCA TCT TTT AAC TGG CAG CAC AAC   645
GGC AGG CAA ATG TAT GTG GCA TTG AAT GGA AAA GGA GCT CCC AGG AGA   693
GGA CAA AAA ACA AGA AGG AAA AAC ACC TCC GCT CAC TTC CTC CCC ATG   741
GTG GTC CAC TCA TAGAAGA AGGCACCGTT GGTGGATGCA GTACAACCAA TGACTCTTTG 800
CCAA
```

[0038]
sequence number: 4
sequence length: 690 bp
sequence form: nucleic acid
number of chain: double-stranded chain
topology: linear chain
sequence type: cDNA
origin
species name: human
sequence:

```
CTTCCAGTAT GTTCCTTCTG ATGAGACAAT TTCCAGTGCC GAGAGTTCCA GTACA ATG   58
TGG AAA TGG ATA CTG ACA CAT TGT GCC TCA GCC TTT CCC CAC CTG CCC  106
GGC TGC TGC TGC TGC TGC TTT TTG TTG CTG TTC TTG GTG TCT TCC GTC  154
CCT GTC ACC TGC CAA GCC CTT GGT CAG GAC ATG GTG TCA CCA GAG GCC  202
ACC AAC TCT TCT TCC TCC TCC TTC TCC TCT CCT TCC AGC GCG GGA AGG  250
CAT GTG CGG AGC TAC AAT CAC CTT CAA GGA GAT GTC CGC TGG AGA AAG  298
CTA TTC TCT TTC ACC AAG TAC TTT CTC AAG ATT GAG AAG AAC GGG AAG  346
GTC AGC GGG ACC AAG AAG GAG AAC TGC CCG TAC AGC ATC CTG GAG ATA  394
ACA TCA GTA GAA ATC GGA GTT GTT GCC GTC AAA GCC ATT AAC AGC AAC  442
TAT TAC TTA GCC ATG AAC AAG AAG GGG AAA CTC TAT GGC TCA AAA GAA  490
TTT AAC AAT GAC TGT AAG CTG AAG GAG AGG ATA GAG GAA AAT GGA TAC  538
AAT ACC TAT GCA TCA TTT AAC TGG CAG CAT AAT GGG AGG CAA ATG TAT  586
GTG GCA TTG AAT GGA AAA GGA GCT CCA AGG AGA GGA CAG AAA ACA CGA  634
AGG AAA AAC ACC TCT GCT CAC TTT CTT CCA ATG GTG GTA CAC TCA TAGAG 684
GAAGGC                                                           690
```

[0039]
sequence number: 5
sequence length: 22 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GATGCATAGG TATTGTATCC AT

sequence number: 6
sequence length: 21 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

TCCATTTTCC TCTATCCTCT C

sequence number: 7
sequence length: 20 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

AGAAGGGGAA ACTCTATGCC

sequence number: 8
sequence length: 21 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GACTGTAAAC TGAAAGAGAG G

sequence number: 9
sequence length: 32 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GCGAGCTCAA GCTTTTTTTT TTTTTTTTTT TT

sequence number: 10
sequence length: 18 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GCGAGCTCAA GCTTTTTT

sequence number: 11
sequence length: 20 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence

CTTCCAGTAT CATCCTTCTG

sequence number: 12
sequence length: 20 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GGCAAAGAGT CATTGGTTGT

sequence number: 13
sequence length: 22 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GATGCATAGG TATTGTATCC AT

sequence number: 14
sequence length: 22 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GAAACTCTAT GGCTCAAAAG AA

sequence number: 15
sequence length: 20 bp
sequence form: nucleic acid
number of chain: single-stranded chain
topology: linear chain
sequence:

GTACACTCAT AGAGGAAGCC

[0040]

[Brief Legends to the Figures]

[Figure 1]

It shows two primers used for the cloning of FGF-10, which are common to FGF-3, FGF-7 and FGF-10: (A) Tyr-Leu-Ala-Met-Asn-Lys; (B) Tyr-Asn-Thr-Tyr-Ala-Ser.

[Figure 2]

It shows primers used for the isolation of FGF-10 cDNA by the Rapid Amplification of cDNA Ends (RACE) method.

[Figure 3]

It shows a summary of the construction of plasmids from plasmid pFGF-10 to plasmid pCDM8-F10SP and finally to plasmid pCDM8-F10HX.

[Figure 4]

It shows primers and PCR conditions used to convert the sequence upstream of the translation start site to the Kozak consensus sequence.

[Figure 5]

It shows the expression of FGF-10 mRNA in articular tissue detected by in-situ hybridization: (A) micrograph of an articular cartilage specimen; (B) micrograph of an apophysiary cartilage specimen.

[Figure 6]

It shows a graph of the incorporation of tritium-labeled thymidine into FRSK cells. Bq, Sp and Hx of the horizontal axis represent control and supernatant samples of FGF-10-

expressing COS cell culture, respectively, while the vertical axis represents the cell-associated radioactivity.

[Document name] Figures

[Figure 1]

sense primer

```
     Tyr Leu Ala Met Asn Lys
5'-  TAC CTA GCA ATG AAC AA -3'
       T   C   C       T
           G   G
           T   T
```

antisense primer

```
     Tyr Asn Thr Tyr Ala Ser
3'-  ATA TTA TGA ATA CGA AG  -5'
       G   G   C   G   C
               G       G
               T       T
```

[Figure 2]

5' RACE method

A: 5'-CCT CTC TTT CAG TTT ACA GTC -3'

A':5'-GAT GCA TAG GTA TTG TAT CCA T-3'

B: 5'-TCC ATT TTC CTC TAT CCT CTC -3'

X: 5'-GCG AGC TCA AGC TTT TTT TTT TTT TTT TTT TT-3'

Y: 5'-GCG AGC TCA AGC TTT TTT -3'

3' RACE method

C: 5'-AGA AGG GGA AAC TCT ATG GC -3'

D: 5'-GAC TGT AAA CTG AAA GAG AGG -3'

D':5'-GAA ACT CTA TGG CTC AAA AGA A-3'

X: 5'-GCG AGC TCA AGC TTT TTT TTT TTT TTT TTT TT-3'

Y: 5'-GCG AGC TCA AGC TTT TTT -3'

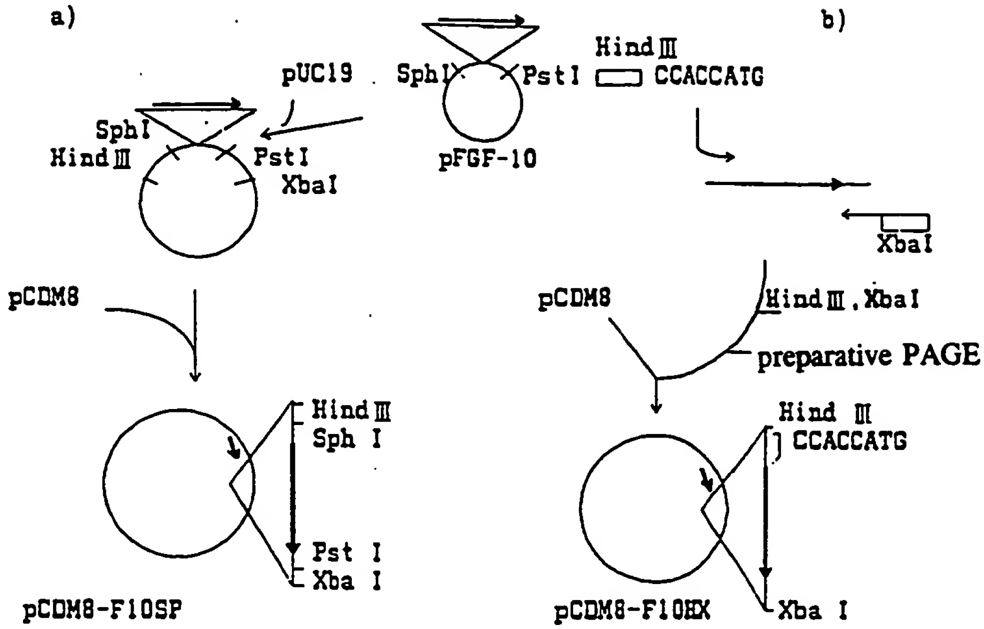
for amplifying total sequence

E: 5'-CTT CCA GTA TGT TCC TTC TG-3'

F: 5'-GGC AAA GAG TCA TTG GTT GT-3'

F':5'-GTA CAC TCA TAG AGG AAG GC-3'

[Figure 3]



a)
b)
pUC19
SphI
PstI
HindIII
CCACCATG
SphI
HindIII
PstI
XbaI
pFGF-10
XbaI
pCDM8
pCDM8
HindIII, XbaI
preparative PAGE
HindIII
SphI
PstI
XbaI
HindIII
CCACCATG
pCDM8-F10SP
pCDM8-F10HX
XbaI

[Figure 4]

Nucleotide sequences of the primers used for converting the region upstream of the deduced translation start site to Kozak consensus sequence

name	number of nucleotide	sequence (5'→3')
F10HS	35mer	TTTTAAGCTT CCACC ATGTGGAAGTGGATACTGAC
FIOXR	27mer	AAAATCTAGA GTCATTGGTTGTACTGC

reaction conditions:

pFGF-10(0.5 μg/μl)	2	μl
10xPCR buffer	10	
10 μM F10HS	2.5	
10 μM F10XR	2.5	
dNTP mix(TaKaRa)	8	
dH2O	74.5	
AmpliTaq	0.5	/100 μl

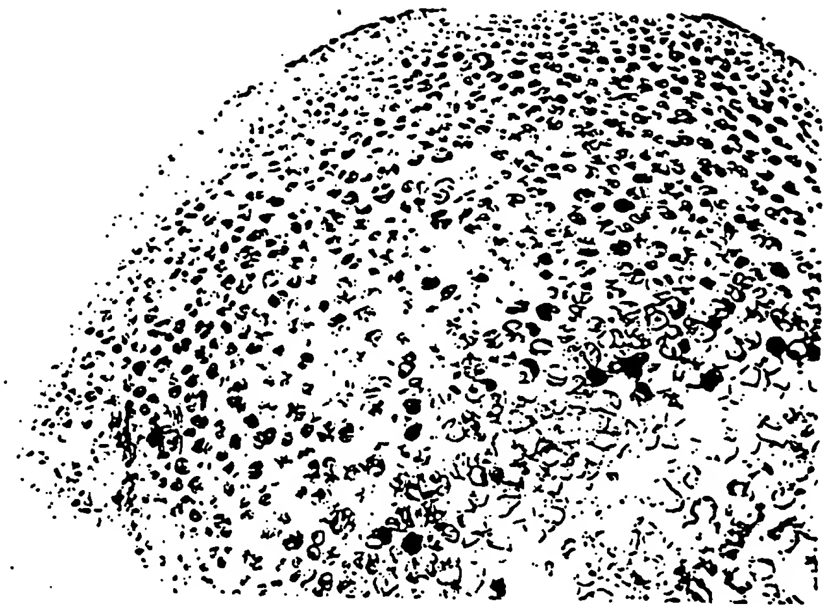
94℃ 30 sec

94℃ 60 sec
56℃ 60 sec
72℃ 60 sec
x 10回

72℃ 9 minutes

mutation-introducing fragments

[Figure 5]

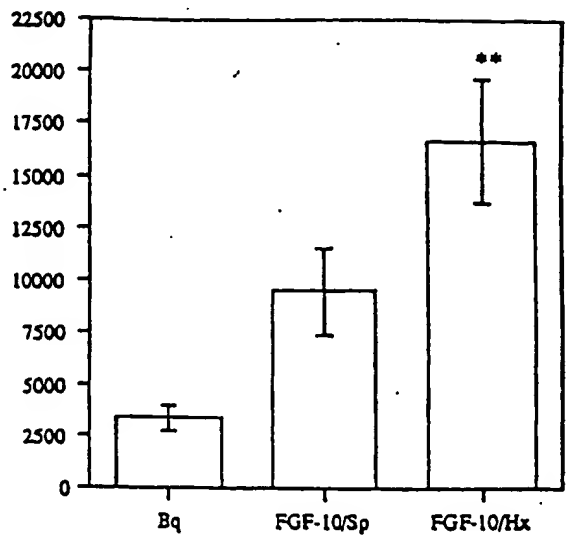


(A) photomicrograph of ultrathin slice specimen of joint cartilage tissue

(B) photomicrograph of ultrathin slice specimen of apophysis cartilage tissue

[Figure 6]

Incorporation of tritium-labeled thymidine (cpm)



(n=6)

COS cell supernatant

Changes of the incorporation of tritium-labeled thymidine in FRSK cells after the addition of COS cell supernatant

Values are mean ± standard deviations. $p<0.01$ is considered significant (**), compared to Bq.

Reference number: 132293

[Document name] Summary

[Summary]

[Subject]

To provide a DNA encoding the fibroblast growth factor FGF-10 having a novel amino acid sequence, the recombinant protein, and method for producing the same.

[Composition]

Recombinant FGF-10 is obtained by transforming an expression vector containing the DNA encoding the specific amino acid sequence into a host cell then culturing the resultant transformant to produce the protein.

[Effect]

FGF-10 can be used as a therapeutic and research agent based on its cell-growth-promoting activity.

[Selection Figures] None.

[Document name] Correction data.
[Corrected document] Patent Application.

<Acknowledged information/additional information>
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